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Important amino acids for the function of the human MT1 melatonin receptor

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Abstract

Models of G protein-coupled melatonin receptor structure suggest that ligand recognition occurs in a binding pocket formed by transmembrane helices III, V and VII. Constitutively active mutations in G protein-coupled receptors have revealed that transmembrane helix III/intracellular loop 2 interface and transmembrane domain VI are critical regions in receptor activation. In this study, nine site-directed mutants of the human MT1 melatonin receptor were created to test the importance of specific amino acids in these regions in ligand recognition and receptor activation events. We analyzed ligand binding, G protein activation and subcellular localization of MT1 receptors transiently expressed in COS-7 cells. Receptor ELISA was employed to study expression levels of N-terminally HA epitope tagged wild-type and mutant MT1 receptors. Mutations in histidine H195 ($His^{5.46}$) in transmembrane domain V reduced receptor affinity for 2-[^{125}I]iodomelatonin. Several other mutants had diminished expression on the plasma membrane. Amino acids M107 ($Met^{3.32}$) in transmembrane domain III and S280 ($Ser^{7.46}$) in transmembrane domain VII were found not to participate in ligand recognition in human MT1 receptor. Constitutive activity was not obtained with mutations in N124 ($Asn^{3.49}$) or P253 ($Pro^{6.50}$). These mutants failed to bind 2-[^{125}I]iodomelatonin and had reduced expression levels. The need to upgrade current melatonin receptor models has become evident. Several important amino acids for the human MT1 melatonin receptor function were revealed in the current study, with effects of mutations ranging from slightly reduced affinity or efficacy to complete loss of function.

Keywords: Constitutively active; G protein-coupled receptor; Melatonin receptor; Receptor ELISA; Rhodopsin-based model; Site-directed mutagenesis

1. Introduction

Melatonin is the major hormonal product of the pineal gland. It is synthesized and secreted during the hours of darkness to regulate the biological rhythms of various endocrine and nonendocrine tissues (for review, see Ref. [1]). The cloning of genes encoding melatonin receptors has revealed the existence of three G protein-coupled melatonin receptor subtypes [2] of which MT1 and MT2 subtypes are expressed in human and other mammals and Mel1c only in amphibia, fish and birds [3–5]. An orphan receptor with no known ligand or function, classified as a member of the melatonin receptor subfamily, has also been cloned from several species [6]. This melatonin-related orphan receptor does not bind 2-[^{125}I]iodomelatonin (^{125}I-Mel) or [^{3}H]melatonin despite its high homology to melatonin receptors.

The information of the primary structures of melatonin receptors facilitated the construction of a three-dimensional rhodopsin-based model for melatonin recognition at its receptor [7]. This model suggests melatonin recognition to occur through specific conserved amino acids in melatonin receptor transmembrane helices TM V–VII, and possibly in TM III. This model has been tested in MT1 receptor by a site-directed mutagenesis study where the mutated receptors were expressed in yeast cells and

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Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GPCR(s), G protein-coupled receptor(s); [^{35}S]GTPγS, guanosine-5′-*O*-(3-[^{35}S]-thio)triphosphate; HA, hemagglutinin; 5-HT, 5-hydroxytryptamine (serotonin); I-Mel, iodomelatonin; ^{125}I-Mel, 2-[^{125}I]iodomelatonin; PBS, phosphate-buffered saline; TM, transmembrane domain; WT, wild-type.

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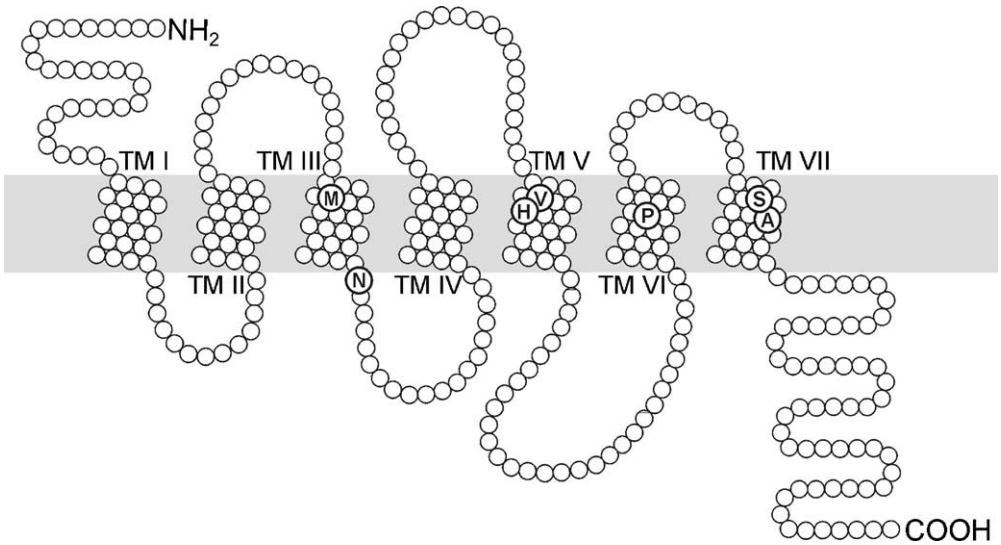

Fig. 1. Schematic illustration of the human WT MT1 melatonin receptor showing the seven transmembrane helices (TM I–VII). Amino acid residues mutated in this study are shown inside larger circles.

receptor activation was measured with a sensitive colorimetric bioassay [8]. This study and other studies with mutated receptors have indicated that a conserved histidine residue in TM V is involved in the interaction between melatonin and the MT1 receptor [8,9]. Amino acids in TM III and VI of the MT1 receptor have also been identified to participate in ligand recognition [10–12].

In this study we analyzed ligand binding, G protein activation and receptor localization of the wild-type (WT) and nine site-directed mutant human MT1 melatonin receptors. (For the location and identification of the mutations, see Fig. 1 and Table 1.) We tested seven mutant receptors that were constructed to address specific questions about the validity of the rhodopsin-based melatonin receptor model [7]. Our second aim was to find out the effect of mutations in two receptor regions on the constitutive activity and G protein activation of the human MT1 melatonin receptor. We mutated a conserved asparagine in the melatonin receptor consensus motif NRY at the intracellular end of TM III into alanine, and a conserved proline in TM VI into alanine. Corresponding mutations have been found to mediate constitutive activity in other G protein-coupled receptors (GPCRs) [13–17]. Constitutively active GPCRs mimic the active conformation of the receptor and are able to activate G proteins in the absence of agonists [18,19].

The human melatonin-related orphan receptor was also included in the studies. A site-directed mutant in TM VII of the melatonin-related receptor was created as an attempt to restore its melatonin binding capacity.

2. Materials and methods

2.1. Materials

Cell culture media and sera were from Gibco and antibiotics from NordCell and EuroClone. ^{125}I-Mel (specific

Table 1
Human MT1 receptors tested in COS-7 cells in this study

Name of the receptor	Mutated amino acid(s)	B_{max} (pmol/mg protein)
WT	None	2.40 ± 0.31
M107D	Met 107 (3.32) into Asp	<0.03
M107T	Met 107 (3.32) into Thr	2.34 ± 0.10
N124A	Asn 124 (3.49) into Ala	<0.03
V192T + H195A	Val 192 (5.43) into Thr and His 195 (5.46) into Ala	0.00
H195A	His 195 (5.46) into Ala	4.79 ± 1.43
H195A + S280A	His 195 (5.46) into Ala and Ser 280 (7.46) into Ala	17.2 ± 5.3*
P253A	Pro 253 (6.50) into Ala	<0.03
S280A	Ser 280 (7.46) into Ala	2.51 ± 0.37
S280F + A284G	Ser 280 (7.46) into Phe and Ala 284 (7.50) into Gly	<0.03

Amino acid numbering counts the residues starting from the first residue in the natural human MT1 receptor. For comparison, the numbering scheme of Ballesteros and Weinstein [25] is shown in parentheses. B_{max} values are mean ± SE and were calculated from homologous competition assays with I-Mel (N = 3–4).

* $P \leq 0.05$ by Tukey's multiple comparison test.

activity 2200 Ci/mmol) was synthesized as described previously [20]. Guanosine-5′-*O*-(3-[^{35}S]-thio)triphosphate ([^{35}S]GTPγS) was purchased from DuPont-NEN and luzindole (*N*-[2-[2-(phenylmethyl)-1*H*-indol-3-yl]ethyl]acetamide) from Tocris. Adenosine deaminase and antibodies were from Roche. Triton X-100 was from Riedel-de Haën. Protein concentrations were determined with Bio-Rad protein assay. Other chemicals were purchased from Sigma or Merck.

2.2. DNA constructs

All mutations were created with QuickChange Site-Directed Mutagenesis Kit (Stratagene). Mutagenic oligonucleotides were used according to manufacturer's instructions to introduce single or double amino acid changes (Table 1) into plasmid pcDNA3 (Invitrogen) containing the coding region of the human MT1 melatonin receptor cDNA (gift from Jason Brown, GlaxoSmithKline), referred as the WT receptor in this paper. Mutations were confirmed by dideoxy sequencing. To allow for the detection of receptor protein in immunological assays, a triple hemagglutinin (HA) epitope tag (gift from Dr. Julia White, GlaxoSmithKline) was subcloned after the initiating Met codon of the WT and mutant MT1 receptors. Triple HA tag contains three nine-amino acid sequences (YPYDVPDYA) derived from the influenza virus HA protein and has higher affinity to antibodies than a single HA tag. The same triple epitope tag was also subcloned after the initiation codon of the human melatonin-related receptor cDNA in pcDNA3 (gift from Jason Brown, GlaxoSmithKline). The melatonin-related receptor was mutated using the same protocol as with the MT1 receptor.

2.3. Cell culture and transient transfection

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS, 100 unit/mL penicillin and 100 μg/mL streptomycin at 37° in humidified atmosphere of 5% CO_2/95% air. For ligand binding assays, 2×10^6 cells were seeded into 175 cm^2 flasks, and for immunoassays, 2×10^6 cells were seeded into 9-cm dishes. Transfections were performed 24 hr later by DEAE-dextran method [21] using 20 or 4 μg of plasmid DNA, respectively. For ligand binding assays and receptor ELISA, at least two independent transfections were analyzed.

2.4. Ligand binding assays

For ^{125}I-Mel binding assays, COS-7 monolayers were washed with and harvested in PBS containing 5 mM EDTA 72 hr after transfections. Cell suspension was aliquoted and pelleted by centrifugation for 5 min at 1600 *g* at 4°. The dry cell pellets were stored at −75°. Cells were resuspended in binding buffer (50 mM Tris–HCl, 2 mM $MgCl_2$, 1 mM EDTA, pH 7.4) at the final concentration of 10 μg/mL and incubated with ^{125}I-Mel with or without competing drugs in a total incubation volume of 250 μL. The reactions were incubated at 25° for 90 min with constant shaking and terminated by the addition of 4 mL of ice-cold wash buffer (50 mM Tris–HCl, pH 7.4), followed by rapid filtration through Whatman GF/B glass fiber filters. After two more 4 mL washes, filters were placed in polypropylene tubes and the radioactivity was measured with Wallac Rackgamma counter. All determinations were done in triplicate. Binding data were analyzed with GraphPad Prism software (GraphPad) using nonlinear regression analysis.

2.5. [^{35}S]GTPγS binding assays

COS-7 monolayers were washed with and harvested in PBS containing 5 mM EDTA 72 hr after transfections. The membranes were prepared and the incubations carried out essentially as previously described [22,23]. Briefly, the assay was performed in duplicate in a final assay volume of 400 μL. The reaction was initiated by adding 40 μL of membrane preparation (5 μg protein/tube) to incubation tubes containing drug dilutions and binding cocktail. The final concentrations of the components in binding reaction were 55 mM Tris–HCl (pH 7.4), 1.1 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$, 0.5% (w/v) BSA, 10 μM GDP, 0.5 unit/mL adenosine deaminase and 150 pM [^{35}S]GTPγS. Nonspecific binding was defined using 10 μM [^{35}S]GTPγS. Reaction tubes were incubated for 90 min at 25° under constant shaking. Reaction was quickly terminated by the addition of 4 mL ice-cold wash buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, pH 7.4) followed by rapid filtration through Whatman GF/B glass fiber filters and two additional 4 mL washes with the buffer. Radioactivity in filters was counted with Wallac Rackbeta liquid scintillation counter. Binding data were analyzed with GraphPad Prism software (GraphPad) using nonlinear fitting for sigmoid dose–response curves.

2.6. ELISA

This indirect immunoassay was used to quantitate the number of HA-tagged receptors on the plasma membrane (intact cells) and the total number of the HA-tagged receptors in the cell (permeabilized cells) with slight modifications of a previously reported protocol [24]. One day after transfections, COS-7 cells were transferred into 96-well plates at the density of 4×10^4 cells/well. About 48 hr later, cells were briefly washed with 0.01 M PBS (pH 7.4) and fixed with 4% (w/v) paraformaldehyde in PBS for 30 min at room temperature. At this stage, if needed, cells were permeabilized with 0.5% Triton X-100 in PBS for 10 min. After two 5 min washes, non-specific binding was blocked with blocking solution (DMEM containing 10% FCS) for 30 min at room temperature. Cells were then incubated with 10 μg/mL

of mouse anti-HA monoclonal antibody (12CA5) in blocking solution for 2 hr at 37°. After two 15 min washes, cells were incubated with 1:5000 dilution of peroxidase-conjugated sheep anti-mouse IgG antibody (Fab fragments) in blocking solution for 1 hr at 37°. The plates were washed twice for 15 min with 0.1 M PBS (pH 6.0) and the enzymatic reaction was started by adding 100 μL of 0.1 M PBS (pH 6.0) containing 4 mM H_2O_2 and 4 mM *o*-phenylenediamine. Color development was stopped with 50 μL 2.25 M H_2SO_4 after 10–16 min incubation at room temperature and the plates were quantified using a plate reader (Molecular Devices) at 490 nm.

2.7. Immunofluorescence microscopy

Confocal immunofluorescence microscopy was employed to study the subcellular distribution of HA-tagged receptors [24]. One day after transfections, COS-7 cells were transferred, at the density of 1×10^5 cells/well, into 6-well plates containing sterile glass coverslips. About 48 hr later, wells containing coverslips were briefly washed with 0.01 M PBS (pH 7.4) and fixed with 4% (w/v) paraformaldehyde in PBS for 30 min at room temperature. At this stage, if needed, cells were permeabilized with 0.5% Triton X-100 in PBS for 10 min. After two 5 min washes, nonspecific binding was blocked with blocking solution (DMEM containing 10% FCS) for 30 min at room temperature. Cells were then incubated with 10 μg/mL of mouse anti-HA monoclonal antibody (12CA5) in blocking solution for 2 hr at 37°. After two 15 min washes, cells were incubated with 1:40 dilution of fluorescein-conjugated sheep anti-mouse IgG antibody in blocking solution for 1 hr at 37°. The coverslips were washed twice with PBS and mounted on microscope slides using 8:1 glycerol/PBS mixture. Images were obtained using a confocal laser-scanning microscope (Leica CLSM).

3. Results

3.1. Immunological detection of MT1 constructs in COS-7 cells

The quantity of the WT and mutant HA-tagged MT1 receptors in transiently transfected COS-7 cells was measured using an indirect ELISA method, in which an increase in receptor number produces an increase in absorbance. Assays with intact cells were performed to quantify N-terminally tagged receptors on the plasma membrane and assays with permeabilized cells were performed to measure the total number of tagged receptors in the cell. The background absorbance (cells transfected with nontagged WT MT1 construct) was 30 and 50% of the absorbance with tagged WT MT1 for intact and permeabilized cells, respectively.

For H195A + S280A cells, the ELISA absorbance in intact cells was slightly higher than for WT MT1 cells (Fig. 2), indicating increased receptor number on the cell surface. The absorbances for M107T or S280F + A284G cells did not differ significantly from the absorbance of WT MT1 cells. In intact cells, the absorbances of the rest of the tested cells expressing receptor mutants were significantly less than the absorbance of the cells expressing WT MT1 receptor. A similar but weaker trend could be seen in permeabilized cells. For all receptors, the absorbances clearly differed from control, indicating that the receptors were expressed on the cell surface and inside the cell.

Immunofluorescence microscopy with permeabilized COS-7 cells showed diffuse staining for the HA-tagged WT MT1 receptors throughout the cell, excluding the nucleus (Fig. 3). In intact (nonpermeabilized) cells only the cell membrane was stained. In permeabilized cells transfected with the double mutant S280F + A284G,

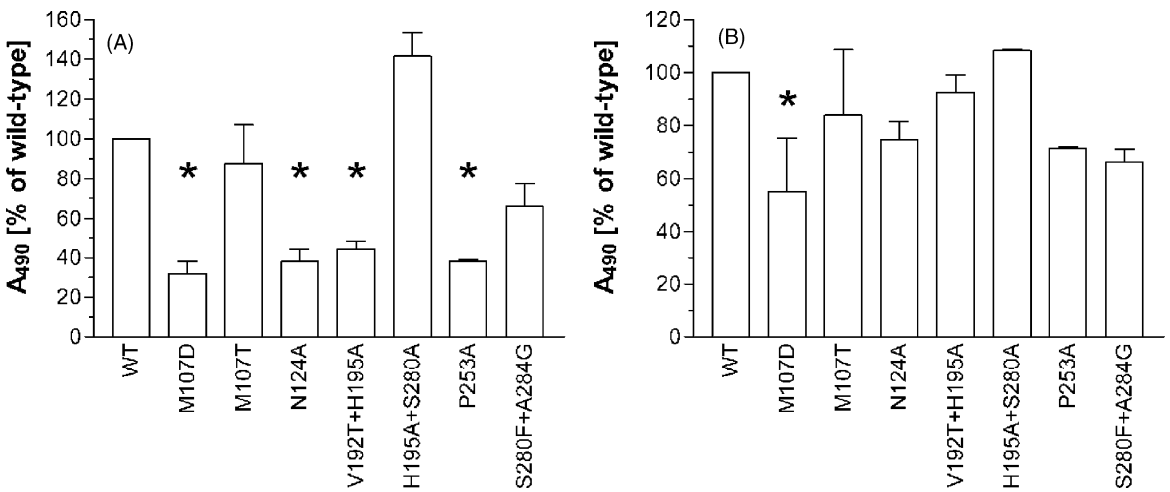

Fig. 2. Relative expression levels of HA-tagged MT1 receptors expressed in COS-7 cells. Background absorbance (cells expressing nontagged version of WT MT1 receptor) has been subtracted. WT expression level = 100%. Results are mean ± SD from two independent transfections. (*) $P \leq 0.05$ by Tukey's multiple comparison test. (A) Intact cells. (B) Cells permeabilized with Triton X-100.

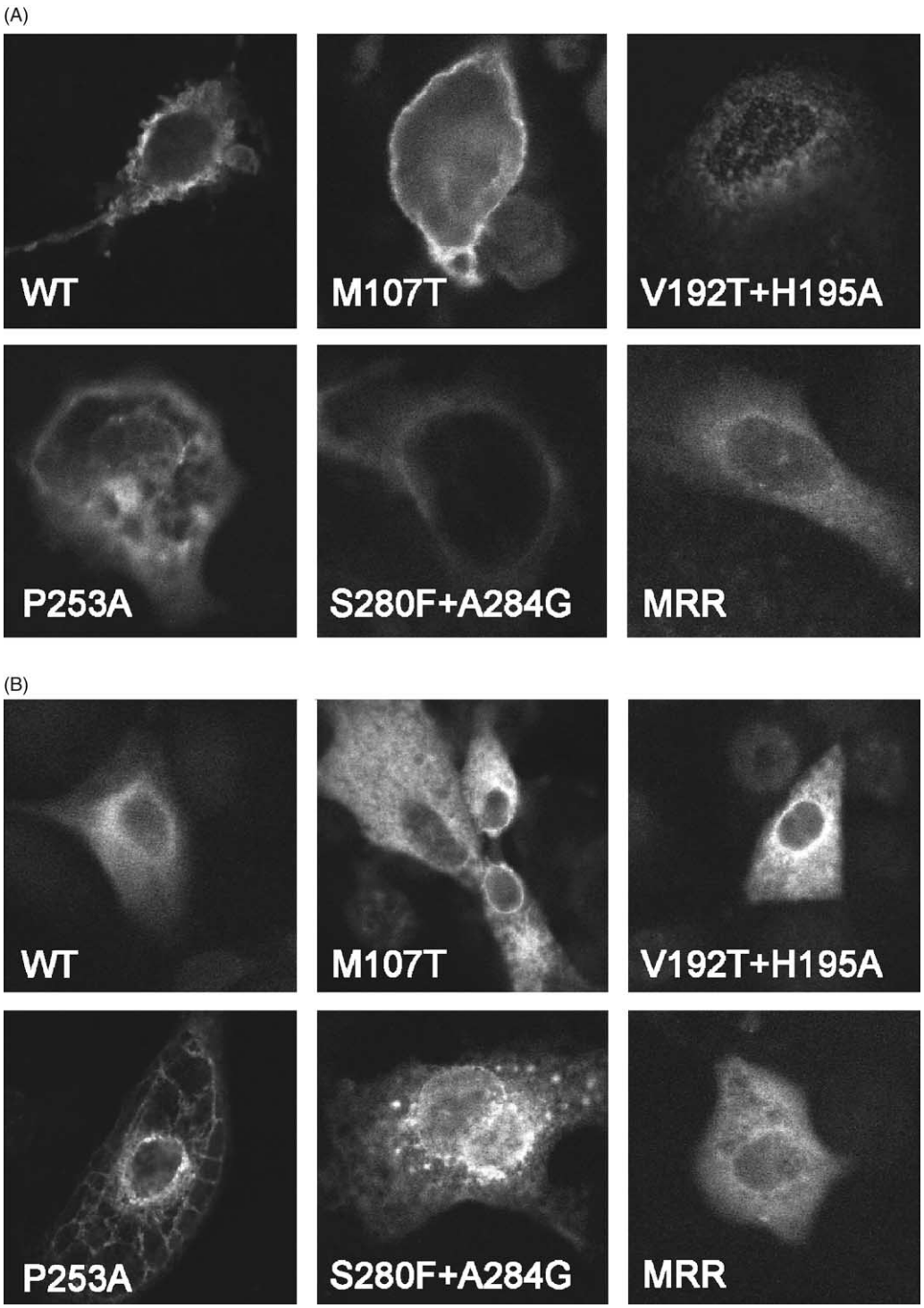

Fig. 3. Confocal microscopic images of COS-7 cells transiently expressing N-terminally tagged MT1 and melatonin-related receptors. (A) Intact cells. (B) Cells permeabilized with Triton X-100. WT = wild-type MT1; MRR = wild-type melatonin-related receptor.

tagged receptor protein showed a different location pattern with receptor seemingly concentrated in intracellular organelles (Fig. 3). HA-tagged mutant P253A seemed to be retained in the endoplasmic reticulum. No differences in staining patterns were detected between the other tested mutant receptors and WT MT1 in permeabilized cells (Fig. 3, and data not shown). In intact cells, there seemed to be less of the M107D, N124A, V192T + H195A and S280F + A284G receptors than of the WT, M107T and H195A + S280A receptors on the cell membrane (Fig. 3, and data not shown).

The HA-tagged WT melatonin-related receptor was also detected with ELISA and confocal microscopy. It was found to be expressed both on the cell surface and inside the cell, in low but detectable levels. Its localization was comparable to the WT MT1 receptors (Fig. 3).

3.2. Ligand recognition of MT1 constructs

WT and nine site-directed mutant human MT1 receptors were expressed transiently in COS-7 cells (Table 1). In ^{125}I-Mel binding assays, B_{max} value for the WT receptor was 2.40 ± 0.31 pmol/mg protein and log (K_d) value was −10.38 ± 0.10 (K_d = 41.7 pM). In preliminary ^{125}I-Mel binding assays with two concentrations of radioligand, only one receptor (V192T + H195A) did not show any specific binding (data not shown). Four other mutants (M107D, N124A, P253A and S280F + A284G) had very poor ^{125}I-Mel binding ability, which prevented a closer determination of ligand binding parameters. The estimated B_{max} values of these mutants were below 0.03 pmol/mg protein. The remaining four mutants had B_{max} values equal to or greater than the WT MT1 receptor (Table 1).

Competition binding assays with I-Mel and melatonin identified that the binding parameters of mutants M107T and S280A were indistinguishable from those of the WT receptor (Table 2). Mutant H195A had slightly higher B_{max}, K_d and K_i values than the WT MT1. The difference in these values was significant between the WT receptor and the mutant receptor H195A + S280A. H195A had 2-fold B_{max} and about 3-fold K_i values for both I-Mel and melatonin, whereas H195A + S280A had approximately 7-fold increase in B_{max} and 10-fold increase in K_i values, compared to the WT MT1.

The WT and mutant A279S melatonin-related receptors were also transiently expressed in COS-7 cells. Cells expressing WT or the mutant did not show any specific ^{125}I-Mel binding (data not shown).

3.3. Functional properties of MT1 constructs in [^{35}S]GTPγS binding assays

The effects of MT1 receptor mutations on receptor function were measured in [^{35}S]GTPγS binding assays with membranes prepared from COS-7 cells transiently expressing MT1 receptor constructs. This technique allows for measuring the G protein activation, which is the primary response in the signaling pathway. Despite of high level of receptor expression in COS-7 cells, the maximum functional responses remained at very modest level, with highest E_{max} values at 150% of basal G protein activation (Table 3). WT MT1 receptor stimulated [^{35}S]GTPγS binding in a concentration-dependent manner, with pEC_{50} at 9.95 ± 0.22 (EC_{50} = 112 pM) and E_{max} at 148 ± 1% from basal.

There were no significant differences between EC_{50} and E_{max} values of the WT MT1 and mutant receptors M107T and H195A + S280A. Mutant S280A had similar EC_{50} value with the WT receptor, but the functional response was very weak with E_{max} of only 116 ± 3%. The maximum response of the mutant H195A and its pEC_{50} were not affected. The double mutant S280F + A284G gave a modest functional response with E_{max} of 118 ± 0% and pEC_{50} value of 6.43 ± 0.45, which makes it more than three orders of magnitude less responsive for melatonin than the WT MT1 receptor.

The MT1 receptor mutants M107D, N124A, V192T + H195A and P253A did not give any response over basal in [^{35}S]GTPγS binding assays (data not shown). The melatonin receptor antagonist luzindole, showing inverse agonist properties in some test systems [26], did not reduce basal [^{35}S]GTPγS binding of the WT receptor or mutants N124A or P253A in concentrations up to 10 μM, implying that these receptors were not constitutively active.

Table 2
Binding profiles of MT1 receptors expressed in COS-7 cells

	log (K_d)	K_i I-Mel (pM)	K_i Mel (pM)
WT	−10.38 ± 0.10	43.5 ± 11.5	188 ± 18
M107T	−10.53 ± 0.04	30.4 ± 4.7	183 ± 9
H195A	−10.00 ± 0.07*	138 ± 36	554 ± 43
H195A + S280A	−9.69 ± 0.08*	410 ± 81*	1830 ± 710*
S280A	−10.43 ± 0.02	46.4 ± 7.4	154 ± 40

Values are mean ± SE. For homologous competition assays with I-Mel (N = 3–4) and for heterologous competition assays with melatonin (N = 2).
* $P \leq 0.05$ by Tukey's multiple comparison test.

Table 3
Comparison of potencies of MT1 receptors expressed in COS-7 cells to activate G proteins in [^{35}S]-GTPγS binding assays

	log (EC_{50})	E_{max} (%)
WT	−9.95 ± 0.22	148 ± 1
M107T	−10.22 ± 0.06	138 ± 2
H195A	−9.13 ± 0.11	135 ± 8
H195A + S280A	−9.47 ± 0.28	151 ± 2
S280A	−9.49 ± 0.20	116 ± 3*
S280F + A284G	−6.43 ± 0.26*	118 ± 0*

Values are mean ± SE. E_{max} is expressed as percentage over basal with nonspecific binding subtracted, N = 3.
* $P \leq 0.05$ by Tukey's multiple comparison test.

4. Discussion

The testing of the rhodopsin-based melatonin receptor model [7] was initiated utilizing functional assays with the yeast *Saccharomyces cerevisae* [8]. The functional assays in the yeast, despite being rapid and very sensitive, reflect the ability of the receptor to activate yeast endogenous G proteins as a consequence of ligand binding and do not necessarily agree with the receptor affinity or the results of functional assays performed in mammalian systems. Since the rhodopsin-based melatonin receptor model [7] concentrated on the ligand recognition event in melatonin receptors, it was important to test the model in ligand binding assays.

In the current study, valine 192 (Val$^{5.43}$) and histidine 195 (His$^{5.46}$) were replaced with amino acids conserved in

mammalian 5-HT (serotonin) receptor subtypes 1, 5 and 7 to create a double mutant V192T + H195A. Corresponding residues are generally thought to interact with the substituents on the aromatic ring of the ligands for biogenic amine neurotransmitter receptors [27]. We also created a single mutant H195A and a TM V–VII double mutant H195A + S280A, in which both of the most important ligand recognition sites in the melatonin receptor model [7] were mutated. Double mutation V192T + H195A totally extinguished ligand binding and functional activation of the MT1 receptor. Receptor ELISA and confocal microscopy showed that the double mutant was expressed, with low amount of receptor on the cell membrane and higher expression inside the cell. The same double mutant gave no functional response in yeast functional assays [8]. In agreement with our finding, mutagenesis of corresponding valine in the ovine MT1 receptor into alanine or leucine produced a decrease both in affinity towards ^{125}I-Mel and potency to inhibit cAMP production [9]. Alanine in this position was tolerated better than leucine. Mutagenesis of His$^{5.46}$ to phenylalanine or leucine in ovine MT1 receptor produced a decrease in affinity and inhibited functional response of the receptor [9]. In the present study, mutations H195A and H195A + S280A decreased human MT1 receptor affinity and increased B_{max}. The data from ELISA and confocal microscopic images with the mutant H195A + S280A also support the finding that the quantity of this double mutant is increased on the cell membrane. With this double mutant there was also a trend towards an increased EC_{50}, which was more prominent with the single mutant H195A. These results conflict the results from yeast functional assays [8] that showed respective mutations to decrease EC_{50}, quite opposite to mammalian functional assay employed in this study. Yeast and mammalian G proteins probably have differences in their MT1 receptor coupling preferences, since a conformational change caused by H195A mutation that is inhibitory in mammalian cells is stimulatory in yeast cells. This finding suggests that caution should be used in extrapolating results generated in yeast-based systems to the mammalian situation.

It can be concluded that in mammalian expression systems, mutations in H195 (His$^{5.46}$) of MT1 receptors decrease receptor affinity and impair functional response. Based on the functional responses, alanine in this position is tolerated quite well (current study) but phenylalanine or leucine completely inhibit receptor activation [9]. Perhaps melatonin can find an interaction with another histidine-mimicking amino acid in close proximity when histidine is replaced with alanine, which has a short side chain. The bigger amino acids phenylalanine or leucine might block receptor activation due to steric hindrance of their bulky hydrophobic side chains.

V192 (Val$^{5.43}$) can be mutated into alanine without dramatic loss of affinity or functionality but mutation to leucine is more harmful [9] and threonine in this position totally inhibits ligand binding and functional response (current study). The double mutant V192T + H195A in our study was probably also suffering from trafficking defects, and was not transported to the cell membrane normally.

Methionine 107 (Met$^{3.32}$) was mutated into threonine (M107T) or aspartate (M107D). The first mutation changed the methionine in MT1 receptors into threonine in melatonin-related receptors. Aspartate in this position is found in biogenic amine receptors, for example, in 5-HT receptors, where it interacts with the protonated ammonium group of serotonergic ligands [28]. M107 (Met$^{3.32}$) appears not to participate in melatonin receptor activation since the mutant M107T was indistinguishable from the WT receptor in every aspect. The same was true in the yeast system [8]. An acidic aspartate in this position probably caused major structural changes in the receptor, resulting in low expression level that severely impaired receptor function (mutant M107D).

Mutations in TM VII revealed that melatonin binding does not require interaction with serine 280 (Ser$^{7.46}$) in MT1 receptor. This is displayed by the fact that mutation S280A, which eliminated the assumed interaction between melatonin and the hydroxyl group of serine, had no effect on ligand binding parameters or EC_{50}. The only effect of this mutation was to reduce E_{max} in mammalian functional assays. In the yeast, both the potency and magnitude of the functional response with S280A were identical to the WT MT1 [8]. A double mutant in TM VII, S280F + A284G, converted MT1 residues to residues conserved in 5-HT receptors. This mutation was found to be extremely harmful for receptor function in yeast expression system, as it totally prevented melatonin signaling (>100,000-fold increase in EC_{50}, compared with the WT) [8]. In mammalian cells, the signaling capacity of this mutant was profoundly compromised, but melatonin was still able to stimulate the receptor with EC_{50} of about 400 nM (3500-fold increase in EC_{50}), despite the very low expression level of this mutant. This is another proof that the optimum G protein activating conformation of MT1 receptor must be different in yeast and mammalian systems. The subcellular localization of this double mutant differed from the WT MT1 and the receptor number, judging from the results of receptor ELISA, was slightly decreased. The receptor probably was not able to accommodate the bulky phenylalanine residue in the position 280 without structural changes that once again affected receptor transport to the cell membrane. The conserved mutation of alanine 284 (Ala$^{7.50}$) to glycine was not likely the major cause of these effects, since bovine MT1 receptor has glycine in this position [29], although the bovine receptor has not been tested in ligand binding or functional assays yet.

We were not able to analyze ligand binding or create dose–response curves for the MT1 receptor mutant N124A. The mutant N124A had dramatically reduced agonist potencies and, interestingly, a very steep dose–response curve when expressed in the yeast but did not

show any constitutive activity [8]. Asparagine 124 ($Asn^{3.49}$) is part of melatonin receptor family consensus sequence NRY, which distinguishes melatonin receptors from other GPCRs, most of which have a DRY or ERY motif at the corresponding location. Mutations in aspartate of this triplet motif have been found to be important in G protein activation and to result in constitutive activity in the gonadotropin-releasing hormone receptor [13] and in the α_{1B} adrenergic receptor [14], among other receptors [19]. Our inability to obtain any functional response in [^{35}S]GTPγS binding assays is probably due to the low cell membrane expression of this mutant in COS-7 cells. The same mutant expressed stably in murine AtT20 cells failed to bind ^{125}I-Mel and was improperly trafficked [30]. In the same study, MT1 mutants N124L and N124K behaved like mutant N124A, while mutants N124D and N124E showed high affinity ^{125}I-Mel binding, normal trafficking, but deficient functional response [30]. These results emphasize the importance of N124 in melatonin receptor function.

Mutant P253A had low expression on the cell membrane and was probably suffering from serious trafficking defects. It did not give functional response in [^{35}S]GTPγS activity. In the yeast, the mutant P253A did not give any response to melatonin [8]. Corresponding proline ($Pro^{6.50}$) controls the equilibrium between the active and inactive state of the yeast α-factor and **a**-factor receptors [15,16] and constitutive activation of these receptors was obtained by the replacement of $Pro^{6.50}$ with any other amino acid [16]. It has been speculated that the same mechanism would control the activity of other GPCRs [16]. The speculation is contradicted by the results of the current study, and the fact that substitutions affecting $Pro^{6.50}$ in rhodopsin, m3 or m5 muscarinic acetylcholine receptor and C5a receptors reportedly did not result in constitutive activity [31–34]. These four receptors belong to a different GPCR family (class A, rhodopsin-like receptors) from the yeast mating factor receptors that belong to class C (fungal pheromone receptors), and it is possible that $Pro^{6.50}$ could be more important in the activation of class C GPCRs.

The WT melatonin-related receptor was found to be expressed in the cell, also in the cell membrane. The inability of the melatonin-related receptor to bind melatonin and related compounds is thus not likely caused by the lack of translation. We were not able to create any ^{125}I-Mel binding affinity in the melatonin-related receptor by mutating alanine 279 ($Ala^{7.46}$) into serine, found in the corresponding position in melatonin receptors. Others have also shown that a chimeric receptor in which TM VII of human MT1 receptor is replaced with TM VII of human melatonin-related receptor, retains measurable ligand binding and functional response capacity [10,11]. It has also been shown with chimeric receptors that the most important region that discriminates between the melatonin binding MT1 receptor and the melatonin-related orphan receptor actually is TM VI [10,11]. The ligands for the melatonin-related receptor still remain unidentified.

This study reveals several amino acids in the MT1 receptor that are important for melatonin recognition and receptor function. Some of the mutations most likely affect receptor conformation and transport to cell surface, thereby preventing normal binding and signaling. Mutations in histidine H195 in TM V of the human MT1 receptor alter ligand binding and functional parameters of the receptor. Several differences in mutant receptor function were found to exist between yeast and mammalian expression systems. The building blocks of melatonin binding pocket have not yet been definitely determined. Our data give support to the common current view that transmembrane domains (at least TM III, V and VI) are important for high affinity melatonin binding in its GPCR. The recently resolved crystal structure of rhodopsin [35] together with results from this and other melatonin receptor mutagenesis studies should facilitate the construction of improved melatonin receptor models.

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